

FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>117-320</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) <b>09/664,692</b> <small>(To Be Assigned)</small>	
INTERNATIONAL APPLICATION NO. <b>PCT/GB99/00935</b>	INTERNATIONAL FILING DATE <b>25 March 1999</b>	PRIORITY DATE CLAIMED <b>25 March 1998</b>	
TITLE OF INVENTION <b>BACTERIA ATTENUATED BY A NON-REVERTING MUTATION IN EACH OF THE AROC, OMPF AND OMPC GENES, USEFUL AS VACCINES</b>			
APPLICANT(S) FOR DO/EO/US <b>CHATFIELD</b>			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time <b>rather than delay</b> examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date.</p> <p>5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).  <input checked="" type="checkbox"/> has been transmitted by the International Bureau.  <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).  <input type="checkbox"/> have been transmitted by the International Bureau.  <input type="checkbox"/> have not been made; however, the time limit for making such amendments has <b>NOT</b> expired.  <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>			
Items 11. To 16. Below concern document(s) or information included:			
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.  <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information. <b>PTO1449/International Search Report</b></p>			

U.S. APPLICATION NO. 09/664,692  
(To Be Assigned)INTERNATIONAL APPLICATION NO.  
PCT/GB99/00935

534 Rec'd PTO/PTO 25 SEP 2000

ATTORNEY'S DOCKET NUMBER  
117-32017.  The following fees are submitted:**BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):**

- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$970.00
- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$840.00
- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO.....\$690.00
- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$670.00
- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$96.00

**CALCULATIONS PTO USE ONLY****ENTER APPROPRIATE BASIC FEE AMOUNT =**Surcharge of \$130.00 for furnishing the oath or declaration later than  20  30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	16	-20 =	0	X \$18.00
Independent Claims	3	-3 =	0	X \$78.00

MULTIPLE DEPENDENT CLAIM(S) (if applicable) \$260.00 S 0.00

**TOTAL OF ABOVE CALCULATIONS =** S 970.00

Reduction by ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28). 0.00

**SUBTOTAL =** S 970.00Processing fee of \$130.00, for furnishing the English Translation later than  20  30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). + 0.00**TOTAL NATIONAL FEE =** S 970.00

Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property + S 0.00

Fee for Petition to Revive Unintentionally Abandoned Application (\$1210.00 - Small Entity = \$605.00) S 0.00

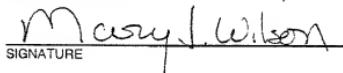
**TOTAL FEES ENCLOSED =** S 970.00

Amount to be: refunded	S
Charged	S

- a.  A check in the amount of \$970.00 to cover the above fees is enclosed.
- b.  Please charge my Deposit Account No. 14-1140 in the amount of S \_\_\_\_\_ to cover the above fees. A duplicate copy of this form is enclosed.
- c.  The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
- d.  The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

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**CHATFIELD**

Atty. Ref.: 117-320

Serial No. (To Be Assigned)

Group:

National Phase of PCT/GB99/00935

Filed: September 25, 2000

Examiner:

For: **BACTERIA ATTENUATED BY A NON-REVERTING  
MUTATION IN EACH OF THE AROC, OMPC AND OMPC  
GENES, USEFUL AS VACCINES**

September 25, 2000

Assistant Commissioner for Patents  
Washington, DC 20231  
Sir:

**PRELIMINARY AMENDMENT**

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

**IN THE CLAIMS**

Claim 6, lines 1-2, change "any one of the preceding claims" to --claim 1--,  
Claim 8, lines 1-2, change "any one of the preceding claims" to --claim 1--,  
Claim 9, lines 1-2, change "any one of the preceding claims" to --claim 1--,  
Claim 10, lines 1-2, change "any one of the preceding claims" to --claim 1--,  
Claim 12, lines 1-2, change "any one of the preceding claims" to --claim 1--,  
Claim 13, line 1, change "any one of claims 1 to 11" to --claim 1--,  
Claim 15, lines 1-2, change "any one of claims 1 to 11" to --claim 1--.

**REMARKS**

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

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## BACTERIA ATTENUATED BY A NON-REVERTING MUTATION IN EACH OF THE AROC, OMPF AND OMPC GENES, USEFUL AS VACCINES

The invention relates to attenuated bacteria useful in vaccines.

5

Background to the invention

The principle behind vaccination is to induce an immune response in the host thus providing protection against subsequent challenge with a pathogen. This may be

10 achieved by inoculation with a live attenuated strain of the pathogen, i.e. a strain having reduced virulence such that it does not cause the disease caused by the virulent pathogen.

15 Clasically, live attenuated vaccine strains of bacteria and viruses have been selected using one of two different methodologies. Mutants have been created either by treatment of the organism using mutagenic chemical compounds or by repeated passage of the organism in

20 vitro. However, use of either method gives rise to attenuated strains in which the mode of attenuation is unclear. These strains are particularly difficult to characterise in terms of possible reversion to the wild type strain as attenuation may reflect single (easily

25 reversible) or multiple mutation events. Furthermore, it is difficult to obtain such strains having optimum immunogenic properties because of multiple mutation events, and multiple strains may need to be used to provide protection against the pathogen.

30

Using modern genetic techniques, it is now possible to construct genetically defined attenuated bacterial strains in which stable attenuating deletions can be created. A number of site directed mutants of *Salmonella* have been 35 created using this type of technology (2, 4, 5, 9, 12,

16, 17, 18). Mutations in a large number of genes have been reported to be attenuating, including the aro genes (e.g. *aroA*, *aroC*, *aroD* and *aroE*), *pur*, *htrA*, *ompR*, *ompF*, *ompC*, *galE*, *cya*, *crp* and *phoP*.

5

Salmonella *aroA* mutants have now been well characterised and have been shown to be excellent live vaccines against salmonellosis in several animal species. In addition, in order to reduce the chances of a reversion to virulence 10 by a recombination event, mutations have been introduced into two independent genes such as *aroA/purA* and *aroA/aroC*. Identical mutations in host adapted strains of Salmonella such as *S.typhi* (man) and *S.dublin* (cattle) has also resulted in the creation of a number of 15 candidate single dose vaccines which have proved successful in clinical (8, 11) and field trials (10).

A *Salmonella typhimurium* strain harboring stable mutations in both *ompC* and *ompF* is described in Chatfield 20 et al (1991, ref. 21). When administered orally to BALB/c mice the strain was attenuated, with the 50% lethal dose (LD50) reduced by approximately 1,000-fold. However, the intravenous LD50 was reduced only by approximately 10-fold, demonstrating the importance of the porins in 25 conferring on the bacteria the ability to infect by the oral route.

Expression of the *ompC* and *ompF* genes is regulated by *ompR*. Pickard et al (1994, ref. 13) describes the cloning 30 of the *ompB* operon, comprising the *ompR* and *envZ* genes, from a *Salmonella typhi* Ty2 cosmid bank and characterisation by DNA sequence analysis. The DNA sequence data were used to identify appropriate restriction sites for generating a defined deletion of

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517 bp within the open reading frame of the *ompR* gene. This deletion was introduced by homologous recombination into the chromosomes of two *S.typhi* strains which already harbored defined deletions in both the *aroC* and *aroD* genes. The *S.typhi* *ompR* mutants displayed a marked decrease in *ompC* and *ompF* porin expression as demonstrated by examination of outer membrane preparations. It was also shown that the *ompR-envZ* two component regulatory system plays an important role in 10 the regulation of Vi polysaccharide synthesis in *S.typhi*.

In animal studies, attenuated *S.typhimurium* has been used as a vehicle for the delivery of heterologous antigens to the immune system (3, 6, 15). This raises the potential 15 of the development of multivalent vaccines for use in man (7).

#### Summary of the Invention

The invention provides a bacterium attenuated by a non-reverting mutation in each of the *aroC* gene, the *ompF* gene and the *ompC* gene. The invention also provides a vaccine containing the bacterium.

It is believed that the *aroC/ompF/ompC* combination of 25 mutations gives a vaccine having superior properties. For example, it is believed that the *aroC/ompF/ompC* combination may be superior to a *aroC/ompR* combination for two reasons:

- 30 1. The *ompR* mutation may cause higher levels of attenuation than the *ompF/ompC* combination of mutations because *ompR* may regulate a number of genes other than *ompF* and *ompC* which are important for survival of the bacterium *in vivo*. Thus, the

*ompF/ompC* combination may allow the bacterium to survive in the vaccinated host for a longer time and at higher levels, resulting in better protection.

5

2. The *ompR* mutation may cause reduced immunogenicity compared to the *ompF/ompC* combination of mutations because *ompR* may regulate the expression of antigens important for immunogenicity.

10

Detailed Description of the Invention

**Bacteria useful in the Invention**

The bacteria that are used to make the vaccines of the invention are generally those that infect by the oral route. The bacteria may be those that invade and grow within eukaryotic cells and/or colonise mucosal surfaces. The bacteria are generally Gram-negative.

- 20 The bacteria may be from the genera *Escherichia*, *Salmonella*, *Vibrio*, *Haemophilus*, *Neisseria*, *Yersinia*, *Bordetella* or *Brucella*. Examples of such bacteria are *Escherichia coli* - a cause of diarrhoea in humans; *Salmonella typhimurium* - the cause of salmonellosis in several animal species; *Salmonella typhi* - the cause of human typhoid; *Salmonella enteritidis* - a cause of food poisoning in humans; *Salmonella choleraesuis* - a cause of salmonellosis in pigs; *Salmonella dublin* - a cause of both a systemic and diarrhoeal disease in cattle, especially of new-born calves; *Haemophilus influenza* - a cause of meningitis; *Neisseria gonorrhoeae* - a cause of gonorrhoea; *Yersinia enterocolitica* - the cause of a spectrum of diseases in humans ranging from gastroenteritis to fatal septicemic disease; *Bordetella*

*pertussis* - the cause of whooping cough; and *Brucella abortus* - a cause of abortion and infertility in cattle and a condition known as undulant fever in humans.

5 Strains of *E.coli* and *Salmonella* are particularly useful in the invention. As well as being vaccines in their own right against infection by *Salmonella*, attenuated *Salmonella* can be used as carriers of heterologous antigens from other organisms to the immune system via  
10 the oral route. *Salmonella* are potent immunogens and are able to stimulate systemic and local cellular and antibody responses. Systems for driving expression of heterologous antigens in *Salmonella* *in vivo* are known; for example the *nirB* and *htrA* promoters are known to be  
15 effective drivers of antigen expression *in vivo*.

The invention may be applied to enterotoxigenic *E.coli* ("ETEC"). ETEC is a class of *E.coli* that cause diarrhoea. They colonise the proximal small intestine.  
20 A standard ETEC strain is ATCC H10407.

Infections of ETEC are the single most frequent cause of travellers diarrhoea, causing 3-9 million cases per year amongst visitors to developing countries. In endemic  
25 areas, ETEC infections are an important cause of dehydrating diarrhoea in infants and young children, resulting in 800,000 deaths a year in the under fives world-wide. In developing countries, the incidence of ETEC infections leading to clinical disease decreases  
30 with age, indicating that immunity to ETEC infection can be acquired. In contrast, naive adults from industrialized countries who visit endemic areas are highly susceptible to ETEC infections. However, with prolonged or repeated visits to endemic areas  
35 susceptibility to ETEC infections diminishes, suggesting

that a live attenuated approach to ETEC vaccination may prove successful.

The inventors chose to work on a non-toxigenic strain of 5 ETEC called E1392/75/2A. E1392/75/2A arose spontaneously from a toxic mutant by deletion of toxin genes. In human studies, oral vaccination with live E1392/75/2A gave 75% protection against challenge with toxin-expressing ETEC from a different serotype. However, approximately 15% of 10 vaccinees experienced diarrhoea as a side effect of the vaccine. The strain needs further attenuation to reduce the side effects before it can be considered as a potential vaccine and the invention gives a means of achieving such attenuation.

15

Seq Id No. 1 shows the sequence of the *E.coli aroC* gene, Seq Id No. 3 shows the sequence of the *E.coli ompC* gene and Seq. Id No. 5 shows the sequence of the *E.coli ompF* gene.

20

**Further mutations**

One or more further mutations may be introduced into the bacteria of the invention to generate strains containing 25 mutations in addition to those in *aroC*, *ompC* and *ompF*. Such a further mutation may be (i) an attenuating mutation in a gene other than *aroC*, *ompC* and *ompF*, (ii) a mutation to provide *in vivo* selection for cells maintaining a plasmid (e.g. a plasmid expressing a 30 heterologous antigen), or (iii) a mutation to prevent expression of a toxin gene.

The further attenuating mutation may be a mutation that is already known to be attenuating. Such mutations

include mutations in aro genes (e.g. *aroA*, *aroD* and *aroE*), *pur*, *htrA*, *ompR*, *galE*, *cya*, *crp*, *phoP* and *surA* (see e.g. refs 2, 4, 5, 9, 12, 13, 16, 17 and 18).

- 5 A mutation to provide selection for maintenance of a plasmid may be made by mutating a gene that is essential for the bacterium to survive. A plasmid carrying the essential gene is then introduced into the bacterium, so that only cells carrying the plasmid can survive. This  
10 may be useful where the plasmid contains, for example, a heterologous antigen to be expressed by the bacterium.

A mutation to prevent expression of a toxin gene may be made to reduce any side-effects caused by vaccination  
15 with the bacterium. For example, in the case of vaccination with *E.coli* strains such as ETEC it may be desirable to mutate the heat labile toxin (LT) or heat stable toxin (ST) genes so that they are not expressed.

20 **The nature of the mutations**

The mutations introduced into the bacterial vaccine generally knock-out the function of the gene completely. This may be achieved either by abolishing synthesis of  
25 any polypeptide at all from the gene or by making a mutation that results in synthesis of non-functional polypeptide. In order to abolish synthesis of polypeptide, either the entire gene or its 5'-end may be deleted. A deletion or insertion within the coding  
30 sequence of a gene may be used to create a gene that synthesises only non-functional polypeptide (e.g. polypeptide that contains only the N-terminal sequence of the wild-type protein).

- The mutations are non-reverting mutations. These are mutations that show essentially no reversion back to the wild-type when the bacterium is used as a vaccine. Such mutations include insertions and deletions. Insertions  
5 and deletions are preferably large, typically at least 10 nucleotides in length, for example from 10 to 600 nucleotides. Preferably, the whole coding sequence is deleted.
- 10 The bacterium used in the vaccine preferably contains only defined mutations, i.e. mutations which are characterised. It is clearly undesirable to use a bacterium which has uncharacterised mutations in its genome as a vaccine because there would be a risk that  
15 the uncharacterised mutations may confer properties on the bacterium that cause undesirable side-effects.
- The attenuating mutations may be introduced by methods well known to those skilled in the art (see ref. 14).  
20 Appropriate methods include cloning the DNA sequence of the wild-type gene into a vector, e.g. a plasmid, and inserting a selectable marker into the cloned DNA sequence or deleting a part of the DNA sequence, resulting in its inactivation. A deletion may be  
25 introduced by, for example, cutting the DNA sequence using restriction enzymes that cut at two points in or just outside the coding sequence and ligating together the two ends in the remaining sequence. A plasmid carrying the inactivated DNA sequence can be transformed  
30 into the bacterium by known techniques such as electroporation and conjugation. It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the bacterium and the wild-type DNA  
35 sequence has been rendered non-functional by homologous

recombination.

**Expression of heterologous antigens**

The attenuated bacterium of the invention may be

- 5 genetically engineered to express an antigen that is not expressed by the native bacterium (a "heterologous antigen"), so that the attenuated bacterium acts as a carrier of the heterologous antigen. The antigen may be from another organism, so that the vaccine provides  
10 protection against the other organism. A multivalent vaccine may be produced which not only provides immunity against the virulent parent of the attenuated bacterium but also provides immunity against the other organism. Furthermore, the attenuated bacterium may be engineered  
15 to express more than one heterologous antigen, in which case the heterologous antigens may be from the same or different organisms.

The heterologous antigen may be a complete protein or a

- 20 part of a protein containing an epitope. The antigen may be from another bacterium, a virus, a yeast or a fungus. More especially, the antigenic sequence may be from *E.coli* (e.g. ETEC), tetanus, hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, herpes  
25 simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus, influenza virus, coxsackie virus or *Chlamydia trachomatis*. Useful antigens include non-toxic components of *E.coli* heat labile toxin, *E.coli* K88 antigens, ETEC colonization factor antigens, P.69 protein  
30 from *B.pertussis* and tetanus toxin fragment C.

The ETEC colonization factors and components thereof are prime candidates for expression as heterologous antigens.

- To instigate diarrhoeal disease, pathogenic strains of  
35 ETEC must be able to colonize the intestine and elaborate

enterotoxins. For most strains of ETEC colonization factors (CF) that are required for adhesion to the intestinal mucosa have been identified. In almost all cases CFs are expressed as fimbriae on the outer surface 5 of the bacteria. A large number of CFs have been identified, the most prevalent being CFAI, CRAII (includes CS1, CS2, CS3) and CFAIV (includes CS4, CS5, CS6).

- 10 A vaccine to ETEC will ideally give protection against a range of colonization factor antigens to ensure that protection against different strains is obtained. In order to achieve this, it would be possible to express several colonization factors in one strain.
- 15 Alternatively, the same attenuations could be made in a range of different ETEC strains, each with a different colonization factor. This would involve deleting the toxins from such strains.
- 20 The DNA encoding the heterologous antigen is expressed from a promoter that is active *in vivo*. Two promoters that have been shown to work well in *Salmonella* are the *nirB* promoter (19, 20) and the *htrA* promoter (20). For expression of the ETEC colonization factor antigens, the 25 wild-type promoters could be used.

A DNA construct comprising the promoter operably linked to DNA encoding the heterologous antigen may be made and transformed into the attenuated bacterium using

- 30 conventional techniques. Transformants containing the DNA construct may be selected, for example by screening for a selectable marker on the construct. Bacteria containing the construct may be grown *in vitro* before being formulated for administration to the host for vaccination 35 purposes.

**Formulation of the vaccine**

The vaccine may be formulated using known techniques for formulating attenuated bacterial vaccines. The vaccine is  
5 advantageously presented for oral administration, for example in a lyophilised encapsulated form. Such capsules may be provided with an enteric coating comprising, for example, Eudragate "S" (Trade Mark), Eudragate "L" (Trade Mark), cellulose acetate, cellulose phthalate or  
10 hydroxypropylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the  
15 bacteria. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal  
20 administration or intramuscular administration.

The vaccine may be used in the vaccination of a mammalian host, particularly a human host but also an animal host. An infection caused by a microorganism, especially a  
25 pathogen, may therefore be prevented by administering an effective dose of a vaccine prepared according to the invention. The dosage employed will ultimately be at the discretion of the physician, but will be dependent on various factors including the size and weight of the host  
30 and the type of vaccine formulated. However, a dosage comprising the oral administration of from  $10^7$  to  $10^{11}$  bacteria per dose may be convenient for a 70 kg adult human host.

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**Examples**

The Examples described in this section serve to illustrate the invention.

**5    Brief description of the drawings**

Figure 1 shows a system for constructing defined deletions in target genes using splicing by overlay extension PCR mutagenesis.

10

Figure 2 shows the expected sequences of target genes after recombination and selection for deletions.

15

Figure 3 shows the cloning of deletion cassettes into plasmid pCVD442.

20

Figure 4 shows an SDS-PAGE analysis of outer membranes prepared from ETEC strains under conditions of low (no salt L-broth) and high (no salt L-broth + 15% sucrose) osmolarity. M = markers; Sample 1 = PTL010; Sample 2 = PTL002; Sample 3 = PTL003; Sample 4 = ΔaroCΔompC; Sample 5 = ΔompF.

25

Figure 5 shows expression of CS1 and CS3 in deletion strains after growth on CFA agar. Equal numbers of cells from each strain were loaded on a 15% SDS-PAGE gel and Western blotted with monospecific anti-CS1 or anti-CS3 polyclonal antibodies. Controls for antibody specificity were whole ces11 lysates of TG1 cells expressing the major pilin protein of CS1, or purified major pilin protein from CS3. Lane M, rainbow low molecular mass markers; lane 1, induced TG1 cells harbouring pKK223; lane 2, induced TG1 cells harbouring pKKCs1; lane 3, CS1-ETEC strain; lane 4, PTL010; lane 5, PTL001; lane 6, PTL002; lane 7, PTL003; lane 8, purified CS3 major pilin

protein.

Figure 6 shows a Southern blot of mutant loci. Chromosomal DNA was extracted from the wild-type ETEC 5 (E1392/75-2A), PTL001 (htrA aroC), PTL002 (aroC ompR) and PTL003 (aroC ompC ompF) as indicated, digested with restriction endonuclease EcoRV, and pulsed field electrophoresed through 1% agarose. DNA was blotted from the gel onto Hybond N+ nylon membranes (Amersham) and 10 hybridised with DNA probes derived from the aroC, htrA, ompR, ompC, or ompF loci as shown. The banding patterns are consistent with the mutant loci being deletions.

Figure 7 shows the IgA responses in volunteers 15 administered a vaccine according to the invention.

**EXAMPLE 1: CONSTRUCTION AND CHARACTERISATION OF STRAIN ACCORDING TO THE INVENTION**

20 Design of deletions and construction of plasmids  
pCVDΔAroC, pCVDΔompC and pCVDΔompF  
Deletions were designated to remove the entire open reading frame of the target gene. Using the *E.coli* genome sequence as a template, PCR primers were designed to 25 amplify fragments of 500-600 base pairs flanking the target open reading frame (see Table 1 for primer sequences). Splicing by overlap extension using PCR was used to fuse the two flanking sequences, creating a PCR product with the entire gene deleted (Figure 1). The 30 wild-type sequences around the deletion site and the predicted sequences after deletion are depicted in Figure 2.

For each gene two different restriction sites were

introduced into the splice region (see Table 2 below). These were used for identification of deletion clones. The PCR primers at either end of the PCR fragment introduced unique restriction sites that were used to 5 clone the fragment into the multiple cloning site of pCVD442 (Figure 3).

PCR products were gel purified using a Qiagen (Trade Name) gel extraction kit and digested with the relevant 10 restriction enzymes prior to ligation to the suicide plasmid pCVD442(22) digested with the same enzyme and treated with alkaline phosphatase to prevent vector self-ligation (Figure 3). The ligation mix was transformed into SY327λpir and plated on L-Ampicillin (100 µg/ml) 15 plates. Plasmids from Ampicillin resistant transformants were screened for the presence of the deletion cassettes by restriction digestion. The following plasmids were generated:

- 20 pCVDΔAroC  
pCVDΔOmpC  
pCVDΔOmpF

The suicide plasmid pCVD442 can only replicate in cells 25 harboring the *pir* gene. On introduction into non-*pir* strains, pCVD442 is unable to replicate, and the Ampicillin resistance conferred by the plasmid can only be maintained if the plasmid is integrated in the chromosome by a single homologous recombination event. 30 The plasmid also has a *sacB* gene, encoding levan sucrase, which is toxic to gram negative bacteria in the presence of sucrose. This can be used to select clones that have undergone a second recombination event, in which the suicide plasmid is excised. Such cells will be resistant 35 to sucrose, but Ampicillin sensitive.

Construction and characterisation of  $\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF strain

This section outlines the chronology of construction and history of a  $\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF strain. In the section,

- 5 "ETEC" refers specifically to strain E1392/75/2A or its derivatives.

$\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF deletions were introduced into E1392/75/2A in the following order:

- 10  $\Delta$ AroC- $\Delta$ AroC $\Delta$ OmpC- $\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF

**Construction of ETEC $\Delta$ AroC**

- 1) E1392/75/2A from original microbanked stock was plated onto L-Agar.
- 15 2) Electroporation competent cells were prepared from these cells. 100  $\mu$ l aliquots were frozen.
- 3) pCVD $\Delta$ AroC was purified from SY327pir cells using a Qiagen Qiafilter (Trade Name) midiprep. The plasmid was concentrated about 10-fold by ethanol precipitation.
- 20 20) The construction of pCVD $\Delta$ AroC is described above.
- 4) 5  $\mu$ l of concentrated plasmid was mixed with 100  $\mu$ l defrosted cells and electroporated. The whole transformation was plated on an L-Ampicillin plate (50  $\mu$ g/ml) and incubated overnight at 37°C.
- 25 5) A single Ampicillin resistant colony grew.
- 6) The colony was streaked onto an L-Ampicillin plate (100  $\mu$ g/ml) and grown overnight at 37°C ("merodiploid plate").
- 30 7) PCR using primers TT19 and TT20 (specific for the aroC gene) and a colony picked from the merodiploid plate amplified two bands, with sizes corresponding to that of the wild-type and  $\Delta$ aroC genes. The sequences of the primers are shown in Table 1

below.

- 8) A colony from the merodiploid plate was grown up for 7 hr in a) L-Ampicillin broth (100 µg/ml) and b) L-Broth. The colony grown on L-Ampicillin was microbanked.
- 5 9) Serial dilutions of the L-broth culture were set up on:

- a) No salt L-agar  
b) No salt L-agar + 5% sucrose.

10 10) The plates were incubated overnight at 30°C.

10) Colony counts showed that  $10^4$  more colonies grew on L-agar than on L-agar + 5% sucrose, showing sucrose selection worked.

11) Sucrose resistant colonies were screened for the presence of  $\Delta$ aroC gene by PCR. Colonies chosen for screening were picked onto an L-agar plate and grown overnight at 37°C. This plate was stored at 4°C, whilst further tests were carried out.

12) 50% of 90 colonies tested had  $\Delta$ aroC only.

20 13) Colonies were tested for growth on:

- a) M-9 minimal media plates  
b) M-9 minimal media + Aromix plates  
c) L-Amp (100 µg/ml)

25  $\Delta$ aroC colonies should not grow on M-9 minimal media without Aromix or on L-Amp.

Aromix is a mix of aromatic compounds as follows:

Substance	Final concentration (% w/v)
Phenylalanine	0.004
Tryptophan	0.004
Tyrosine	0.004
p-aminobenzoic acid	0.001
dihydroxybenzoic acid	0.001

- These compounds are made in wild-type bacteria, but the *aroC* mutation prevents their synthesis.
- 14) 13/14 putative  $\Delta$ AroC colonies required Aromix for growth on M-9 minimal media and were susceptible to Ampicillin.
- 15) 3 colonies (No. 1,2,3) were tested for the presence of the CS1 major pilin protein gene by PCR using primers MGR169 and MGR170. All 3 colonies gave PCR products of the expected size (700 bp.). The sequences of the primers are shown in Table 1.
- 10 16) Colonies 1, 2 and 3 from screening master plate were streaked onto L-Agar and grown overnight at 37°C. Cells from these plates were used to inoculate microbank tubes.
- 15 17) Colony 1, stored in a microbank, was used for further work.
- 18) For permanent storage, a bead from the microbank tray was inoculated into 1 ml L-broth, grown for 4 hr with shaking at 37°C and used to make agar slopes which were used to make freeze dried stocks.
- 20 The freeze dried stock of E1392/75/2 $\Delta$ AroC was designated PTL004. 20 ml of L-broth was added to the rest of the 1 ml culture and the culture was incubated overnight at 30°C. 1 ml of the overnight culture was transferred to each of three cryovials and stored in liquid nitrogen.
- 25

**Construction of ETEC $\Delta$ AroC $\Delta$ OmpC**

- 1) Preparation of pCVDA $\Delta$ OmpC plasmid DNA for electroporation:
- 30 A colony of SY327 $\lambda$ pir harbouring pCVDA $\Delta$ OmpC was grown overnight at 37°C in 100 ml L-Ampicillin broth (100 µg/ml). Plasmid DNA was purified using 2 Qiagen Qiafilter (Trade Name) midipreps. DNA was
- 35

- further concentrated by ethanol precipitation. The construction of pCVDΔ*OmpC* is described above.
- 2) Preparation of electrocompetent cells:  
ETECΔ*AroC* cells from the microbank tray produced in  
5 step 17 of the preceding section were streaked on L-agar, grown at 37°C overnight and then stored at 4°C for no more than 1 week before being used to inoculate cultures for preparing electrocompetent cells.
- 10 3) ETECΔ*AroC* cells were electroporated with 5 µl of concentrated pCVDΔ*OmpC* DNA, and each transformation plated on a single L-Ampicillin plate (50 µg/ml) and grown overnight at 37°C.
- 4) 17 Ampicillin resistant colonies (putative  
15 ETECΔ*AroC*/ pCVDΔ*OmpC* merodiploids) were obtained.
- 5) These colonies were spotted onto a master L-  
Ampicillin (100 µg/ml) plate and used as templates  
for PCR with primers TT7/TT8. The master plate was  
grown at room temperature over the weekend. The  
20 sequences of the primers are given in Table 1  
below.
- 6) A single colony (No. 7) had the *ΔompC* gene.  
7) The colony was grown for 5 hr in L-broth.  
8) Serial dilutions of the L-broth culture were set up  
25 on:  
a) No salt L-agar  
b) No salt L-agar + 5% sucrose.  
The plates were incubated overnight at 30°C.  
9) Colony counts showed that 10<sup>4</sup> more colonies grew on  
30 L-agar than on L-agar + 5% sucrose, showing sucrose  
selection worked.
- 10) 45 sucrose resistant colonies were screened for  
*ΔompC* by PCR using primers TT7 and TT8. 9 colonies  
had the *ΔompC* gene, but most had traces of w.t.  
35 *ompC* gene. The sequences of the primers are given

in Table 1 below.

- 11) To further characterise putative ETECA $\Delta$ AroC $\Delta$ OmpC colonies, they were grown in 1 ml L-Broth for 5 hr and plated on:

- 5           a)   L-Agar + 100 µg/ml Ampicillin  
              b)   L-Agar  
              c)   L-Agar + 5% sucrose

$\Delta$ OmpC colonies should be resistant to sucrose and sensitive to Ampicillin.

- 10 12) Only 1 colony (No. 1) was Ampicillin sensitive and sucrose resistant.

- 13) Colony 1 was checked for the presence of  $\Delta$ aeroC,  $\Delta$ ompC and CS1 genes by PCR with primers TT19/TT20, TT7/TT8 and MGR169 and 170. The sequences of the 15 primers are given in Table 1 below.

- 14) Colony 1 gave single PCR products of the expected size for  $\Delta$ aeroC,  $\Delta$ ompC and CS1 genes.

- 15) The colony was microbanked.

- 16) For permanent storage, a bead from the microbank 20 was inoculated into 1 ml L-broth, grown for 4 hr with shaking at 37°C and used to make agar slopes which were freeze dried. The freeze dried stock of E1392/75/2A $\Delta$ AroC $\Delta$ OmpC was designated PTL008. 20 ml of L-broth was added to the rest of the 1 ml 25 culture and the culture was incubated overnight at 30°C. 1 ml of the overnight culture was transferred to each of three cryovials and stored in liquid nitrogen.

30 **Construction of ETECA $\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF**

Conjugation was used to introduce pCVDA $\Delta$ OmpF into E1392/75/2A $\Delta$ AroC $\Delta$ OmpC.

- 1) Conjugation donor cells SM10λpir were transformed with pCVDA $\Delta$ OmpF. The construction of plasmid

pCVD $\Delta$ OmpF is described above.

- 2) ETEC $\Delta$ AroC $\Delta$ OmpC cells were conjugated with SM10 $\lambda$ pir/  
pCVD $\Delta$ OmpF cells. The pCVD442 plasmid includes a  
transfer origin which allows the plasmid to be  
5 transferred from a donor strain containing the RP4  
transfer genes (e.g. SM10 $\lambda$ pir) to a recipient  
strain (e.g. ETEC). ETEC $\Delta$ a $\Delta$ ompC cells and  
E.coli strain SM10 $\lambda$ pir harbouring the Pcvd $\Delta$ ompF  
recombinant were cross-streaked on L-agar plates so  
10 as to cover an area of approximately 10 cm<sup>2</sup>.  
Plates were incubated at 37°C for 20 h, then the  
growth washed off using 4 ml L-broth and the  
suspension plated onto McConkey agar (Difco)  
15 containing streptomycin at 20 $\mu$ g ml<sup>-1</sup> and ampicillin  
at 300 $\mu$ g ml<sup>-1</sup>. Plates were incubated overnight at  
37°C and resulting colonies were checked for  
merodiploidy by PCR using appropriate  
oligonucleotides as primers.
- 3) Putative ETEC transconjugants were screened. 10  
20 colonies were picked from McConkey agar plates and  
grown overnight on L-Ampicillin (100  $\mu$ g/ml) agar.  
The presence of  $\Delta$ ompF gene was checked for by PCR  
with primers TT1/TT2. The sequences of the primers  
are given in Table 1 below.
- 25 4) The colonies were grown for 5 hr in L-broth.
- 5) Serial dilutions of the L-broth culture were set up  
on:
- a) No salt L-agar
  - b) No salt L-agar + 5% sucrose.
- 30 6) The plates were incubated overnight at 30°C.
- 6) Colony counts showed 10<sup>5</sup> more colonies grew on L-  
agar than on L-agar + 5% sucrose, showing sucrose  
selection worked.
- 7) Sucrose resistant colonies were screened for  $\Delta$ ompF  
35 gene by PCR with primers TT1/TT2. The sequences of

the primers are given in Table 1 below. The screened colonies were grown overnight on L-Agar. 3 colonies out of 47 had the  $\Delta$ ompF gene with no evidence of the wild-type ompF gene.

- 5 8) To further characterise putative ETEC $\Delta$ AroC $\Delta$ OmpC $\Delta$ OmpF colonies, they were plated on:

- a) L-Agar + 100 µg/ml Ampicillin
- b) L-Agar
- c) L-Agar + 5% sucrose

10  $\Delta$ ompF colonies should be resistant to sucrose and sensitive to Ampicillin.

- 9) All three  $\Delta$ ompF colonies were Ampicillin sensitive and sucrose resistant.

- 10) The colonies were microbanked and one colony was chosen as a master stock.

- 15 11) For permanent storage, a bead from the master stock was inoculated into 1 ml L-broth, grown for 4 hr with shaking at 37°C and used to make agar slopes which were used to make freeze dried stocks. The 20 freeze dried stock of E1392/75/ 2A $\Delta$ aroC $\Delta$ ompC $\Delta$ ompF was designated PTL003. 20 ml of L-broth was added to the rest of the 1 ml culture and the culture was incubated overnight at 30°C. 1 ml of the overnight culture was transferred to each of three cryovials 25 and stored in liquid nitrogen.

Characterisation of E1392/75/ 2A $\Delta$ aroC $\Delta$ ompC $\Delta$ ompF

- 1) Growth requirements:

30 Cells taken from the master stock produced in step 10 of the preceding section were streaked on L-Agar plate. At the same time 8 ml L-broth was inoculated for a chromosomal DNA prep for Southern blots. Both plate and liquid culture were grown overnight at 37°C.

35 Cells from the grown plate were streaked onto the

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following media and grown overnight at 37oc.

	<u>Medium</u>	<u>Growth</u>
5	L-Amp	No
	M9 minimal media	No
	M9 minimal + Aromix	Yes
10	M9 + sulfathiazole (100 µg/ml)	No
	M9 + sulfathiazole (100 µg/ml) + Aromix	Yes
	L-Agar + 50 µg/ml streptomycin	Yes
	L-Agar + 5% sucrose	Yes
15	As expected, the cells were Amp sensitive. The cells were resistant to sucrose, streptomycin and sulfathiazole, but required Aromix to grow on minimal media.	
20	2) LPS analysis of PTL003:	
	a) A freeze dried vial of PTL003 was broken open. The culture was resuspended in L-Broth and plated on L-Agar for growth. Some cells were scraped off and stored in microbank.	
25	b) More cells were scraped off and the LPS profile was analysed. There was no visible difference between the LPS profile of PTL003 and original E1392/75/2A strain.	
30	3) Confirmation of deletions by PCR:	
	a) A scrape of cells was taken from the plate made in in 2a and streaked onto L-Agar and grown overnight.	
	b) Freshly grown cells were used for PCR with primers that flank the following genes: <i>aroC</i> , <i>htrA</i> , <i>ompC</i> , <i>ompF</i> , <i>ompR</i> .	
35	c) PTL003 was shown to have deletions in <i>aroC</i> ,	

- ompC* and *ompF* genes, but not in *htrA* or *ompR*.
- 4) Analysis of outer membrane protein profile of PTL003:  
Outer membrane protein fractions were prepared from strains PTL010 (E1392/75/2A) and the deletion strains PTL002 and PTL003. A strain with a single *ompF* deletion and a strain with both *aroC* and *ompC* deletion were also analysed. Strains were grown under conditions of low osmolarity (no salt L-broth) and high osmolarity (no salt L-broth+15% sucrose). The OmpF protein product is normally expressed at low osmolarity whereas the OmpC product is expressed at high osmolarity. The OmpC and OmpF proteins have similar electroporetic mobilities. At both high and low osmolarities, the strain PTL003 lacks proteins in the OmpC/OmpF region when compared to the wild-type E1392/75/2A strain or to the  $\Delta$ AroC $\Delta$ OmpC or  $\Delta$ OmpF deletion strains. The results are shown in Figure 4.
- 5) Expression of CS1 and CS3 pili on CFA agar:  
The expression of CS1 and CS3 pili in the deletion strains was examined. Equal numbers (2  $A_{600nm}$  units) of bacteria strains PTL010, PTL001, PTL002 and PTL003 grown overnight at 37°C on CFA agar were subjected to SDS PAGE and analysed by Western blotting with monospecific polyclonal antibodies against CS1 or CS3. CS1 and CS3 pili were expressed equally well in four strains (Figure 5).
- 30 A CFAII-negative derivative of E1392/75/2A was constructed for use as a control. This was done by specific curing of the CS encoding plasmids from ETEC strain E1392/75-2A. A short fragment of DNA was amplified from the *cooB* gene using PCR with

oligonucleotides CSA01 and CSA02 as primers and  
ligated into pGEM-T Easy plasmid vector (Trade  
Name, Promega) designed for the cloning of PCR  
products. The fragment was subcloned into pCVD442  
5 by virtue of the SalI and SphI restriction enzyme  
sites. The pCVD442-cooB derivative was introduced  
into ETEC strain E1392/75/2A by conjugation from  
SM10λpir. Ampicillin resistant transconjugants are  
most likely to be the result of fusion of the  
10 pCVD442-cooB derivative with cooB-bearing plasmid.  
Such transconjugates were then grown on L-agar  
supplemented with 5% sucrose to select for loss of  
the sacB gene of pCVD442. Resulting colonies were  
tested for ampicillin sensitivity, and by PCR using  
15 CSA01 and CSA02 as primers. Three colonies of  
E1392/75/2A were included as positive controls  
among these PCRs. Two sucrose resistant colonies  
that gave no product with the PCR were streaked out  
onto fresh L-agar supplemented with 5% sucrose to  
20 obtain pure cultures. These were then grown in L-  
broth at 37°C for approximately 16 h and  
microbanked at -70°C. Loss of the CS1 encoding  
plasmid was confirmed by analysis of the plasmid  
profiles of the derivatives using agarose gel  
25 electrophoresis. Two derivatives were confirmed as  
CS1 negative, but were still CS3+.

6) Southern blotting of PTL003:  
Structure of deletion mutations. Total DNA was  
30 extracted from cultures of the three deletion  
mutants grown from the microbanked stocks, digested  
with restriction endonuclease EcoRV, and the  
digested DNA subjected to pulsed field agarose gel  
electrophoresis. DNA was blotted from the gels  
35 onto Hybond N+ (Trade Name) nylon membranes and

hybridised with appropriate DNA probes according to standard procedures. Results (Figure 6) show that the hybridising chromosomal DNA fragments of the mutants are shorter than the wild-type, consistent  
5 with the mutations being deletions.

*Confirmation of absence of Heat-Stable (ST) and Heat-Labile (LT) toxin genes in E.coli strain E1392/75-2A.* For this the ST and LT-AB genes were used as DNA probes against total DNA from E1392/75-2A. Total DNA from the toxin positive ETEC strain E1393/75 was included as a positive control, while that from the laboratory *E.coli* strain JM109 was included as a negative. Hybridised membranes were left under Hyperfilm-ECL (Trade Name) for 1 h to obtain the maximum amount of signal. Probes were prepared using PCR with plasmid DNA extracted from E1392/75-2A as template and oligonucleotides EST01 and EST02 as primers for ST, or LT-R1 and LT-03 for LT-AB. There was no significant hybridisation with total DNA using either the LT-AB or the ST probe, despite obtaining a very intense signal from the positive control total DNA.  
10  
15  
20

*Confirmation of absence of pCVD442 sequences from the chromosome of deletion mutants.* The plasmid pCVD442 was labelled and hybridised to total DNA from deletion mutants PTL001, PTL002 and PTL003 digested with EcoRV. Total DNA from ETEC strain E1392/75-2A was included as a control. A complex pattern of hybridising DNA fragments was obtained. But, there was no significant difference between the pattern obtained for the wild-type and that for the mutants, indicating that probably no residual pCVD442 nucleotide sequences were left in the  
25  
30  
35 genomes of the mutants. The complex pattern of

hybridising fragments was most likely due to the pCVD442 probe hybridising with the plasmid DNA components of the E1392/75-2A strain and mutant derivatives.

5

Table 1 - PCR primers

Name	Target	Use	Sequence (5'-3')
TT1	<i>ompF</i>	Primer A for cloning	ATC TGT TTG TTG AGC TCA GCA ATC TAT TTG CAA CC
TT2	<i>ompF</i>	Primer B for cloning	TTT TTT GCC AGC ATG CCG GCA GCC ACG CGT AGT G
TT3	<i>ompF</i>	Primer C for cloning	CTC GAG GCT TAG CTC TAT TTA TTA CCC TCA TGG
TT4	<i>ompF</i>	Primer D for cloning	GAG CTA AGC CTC GAG TAA TAG CAC ACC TCT TTG
TT7	<i>ompC</i>	Primer A for cloning	TTG CTG GAA AGT CGA CGG ATG TTA ATT ATT TGT G
TT8	<i>ompC</i>	Primer B for cloning	GGC CAA AGC CGA GCT CAT TCA CCA GCG GCC CGA CG
TT9	<i>ompC</i>	Primer C for cloning	GCT AAG CCT CGA GTA ATC TCG ATT GAT ATC CG
TT10	<i>ompC</i>	Primer D for cloning	CTC GAG GCT TAG CGT TAT TAA CCC TCT GTT A

TT19	<i>aroC</i>	Primer A for cloning	CCG CGC TCG CTC TAG AGT GAA CTG ATC AAC AAT A
TT20	<i>aroC</i>	Primer B for cloning	ATG CGC GCG AGA GCT CAA CCA GCG TCG CAC TTT G
TT21	<i>aroC</i>	Primer C for cloning	CTC GAG GCA TGC TGA ATA AAA CCG CGA TTG
TT22	<i>aroC</i>	Primer D for cloning	GCA TGC CCT CGA GGG CTCC GTT ATT GTT GTG
MGR169	CS1	Binds in CS1 sequence	TGA TTC CCT TTG TTG CGA AGG CGA A
MGR170	CS1	Binds in CS1 sequence	ATT AAG ATA CCC AAG TAA TAC TCA A
LT-R1	LT-AB	See text	GCT TTT AAA GGA TCC TAG TT
LT-03	LT-AB	See text	GGT TAT CTT TCC GGA TTG TC
EST01	ST	See text	CAT GTT CCG GAG GTA ATA TGA A
EST02	ST	See text	AGT TCC CTT TAT ATT ATT AAT A
CSA01	CS1	See text	TGG AGT TTA TAT GAA ACT AA
CSA02	CS1	See text	TGA CTT AGT CAG GAT AAT TG
CS3-01	CS3	See text	ATA CTT ATT AAT AGG TCT TT
CS3-02	CS3	See text	TTG TCG AAG TAA TTG TTA TA

Table 2

	Target gene	Sites used for cloning into pCVD442		Sites introduced for screening purposes	
		Site 1	Site 2	Site 3	Site 4
5	aroC	XbaI	SacI	XhoI	SphI
	htrA	SalI	SphI	XhoI	XbaI
10	ompC	SalI	SacI	BspI	XhoI
	ompF	SacI	SphI	BspI	XhoI
	ompR	SalI	SacI	BspI	SphI

**EXAMPLE 2: SAFETY AND IMMUNOGENICITY OF ATTENUATED VACCINE STRAIN OF ENTEROTOXIGENIC *E. COLI* ( $\Delta$ aroC/ $\Delta$ ompC/ $\Delta$ ompF) IN HUMAN VOLUNTEERS**

The study was designed to evaluate a candidate live attenuated vaccine strain of enterotoxigenic *E. coli*, namely the  $\Delta$ aroC/ $\Delta$ ompC/ $\Delta$ ompF PTL003 described above.

20

**Preparation of the vaccine seed lots**

The bacterial strain was plated onto MacConkey agar for 25 purity and for confirmation of identity, and 5 colonies used to inoculate a flask containing 200 ml of luria broth. After 8 hours incubation at +37°C, 30 ml of sterile glycerol was added to the broth culture and aliquots prepared (1 ml per vial). One hundred such vials were 30 frozen at -70°C. These vials constituted the seed lot for the vaccine strain.

Purity of the seed lot was ensured by selecting ten random vials, and testing them for bacterial purity and freedom from fungi. An additional three vials were tested to determine the number of bacteria in the vials using 5 standard plate count methods with serial dilutions of the culture broth.

**Preparation of the vaccine**

10 The vaccine was prepared fresh prior to each vaccination and all steps in the preparation of the inoculum carried out in a safety cabinet. The day prior to vaccination, 0.2 ml was spread onto the surface of luria agar plates using sterile cotton swabs to prepare the lawn of bacteria. The 15 same culture broth was streaked onto MacConkey and luria agar plates for purity. The agar plates were incubated at 37°C for 18 hours in a sealed container with tamper-resistant indicator tape to ensure that the plates were not tampered with during incubation. After incubation, the lawn 20 of bacteria was harvested with 5 ml of sterile phosphate buffered saline (PBS), and the optical density of the suspension measured. The appropriate volume of this suspension, corresponding to the desired dose, was then placed into unit dose bottles with 30 ml of bicarbonate 25 buffer and administered to the volunteers. An extra dose of vaccine was prepared and left in the laboratory, and immediately after the volunteers had been vaccinated the actual number of bacteria in each dose of vaccine was validated using standard colony count procedures with ten 30 fold dilutions of vaccine.

The procedure for diluting the bacteria was established during preliminary studies using lawn cultures prepared and incubated exactly as done for the vaccine preparations 35 administered to volunteers. Suspensions were made and the

number of viable bacteria enumerated by colony counts of serial dilutions and related to the determined optical density. Based on these preliminary studies, a standard procedure was developed for preparing and validating the 5 correct dilutions of bacteria in order to give the doses stated.

**Preparation of buffer**

10 A buffer consisting of sodium bicarbonate in water was used. For each dose of vaccine 150 ml of deionised water containing 2 gram of sodium bicarbonate was prepared and filter sterilised. 30 ml of the buffer was placed into 50 ml sterile vials and the dose of vaccine bacteria was added 15 to these vials. The remaining 120 ml of buffer was placed into separate sterile bottles. At the time of vaccination, the volunteers were first administered 120 ml of buffer, then a minute later, 30 ml of buffer containing the vaccine.

20

**Vaccination schedule**

Groups of volunteers were studied in a dose escalation manner. The first group of volunteers received a dose of 25 approximately  $5 \times 10^7$  bacteria, the second a dose of approximately  $5 \times 10^9$  and the third group a dose of approximately  $5 \times 10^8$ .

The volunteers were given Ciprofloxacin 500 mg BID for 30 three days beginning on day 4. They were discharged on day 6, having had a haematology and chemistry screen for safety. Serum was saved for antibody measurement.

On days 9 and 14 the volunteers returned for follow-up 35 outpatient visits at which time an interval history was

done and a blood sample was obtained for serological assays. In total, blood (40 ml) was collected for serology three times, prior to vaccination and on day 9 and day 14 after vaccination.

5

#### **Laboratory Assay Procedures**

Up to two faecal specimens were cultured each day while the volunteers were in hospital. For qualitative cultures, a 10 faecal swab was placed into Cary Blair transport media and taken to the laboratory where it was inoculated directly onto MacConkey agar and onto MacConkey agar containing antibiotics selective for the vaccine strain. Up to five colonies were shown to be agglutinated using antisera 15 specific for the vaccine strain. For quantitative culture (first specimen each day only) faecal specimens were weighed and diluted in PBS, with serial 10-fold dilutions up to  $10^{-4}$ , and then 100  $\mu$ l of each dilution was spread onto MacConkey agar with antibiotics. Suspected colonies were 20 confirmed by agglutination with anti-O serum.

Serum was collected and saved for subsequent assay for antibody against CFA II antigens by ELISA and bactericidal antibody against the vaccine strain.

25

Peripheral blood mononuclear cells were separated from whole blood collected into citrate and washed. These cells were cultured at a density of  $10^7$  cells per ml in RPMI tissue culture medium at 37°C for 48 hours. After 48 hours 30 the supernatant was transferred to a cryovial and frozen at -20°C until it could be assayed for IgG and IgA antibody to CFA II by ELISA.

Table 3 - Summary of the procedures of the protocol

Day (Vaccination day is day 0)	pre	-1	0	1	2	3	4	5	6	9	14
5 Recruitment / screening	x										
HCG (urine)	x				x						
Training/ consent	x										
10 Inpatient stay		x	x	x	x	x	x	x	x		
Vaccination			x								
Outpatient visit	x									x	x
15 Stool cultures - quantitative		x	x	x	x	x	x	x	x	x	x
Stool cultures - qualitative		x	x	x	x	x	x	x	x	x	x
Serology		x								x	x
CBC/Chem panel	x								x		
20 Ciprofloxacin 500mg BID for 3d							x	x	x		

**Results:**

- 25 No symptoms were seen at all actual doses of  $6.8 \times 10^7$  and  $3.7 \times 10^8$  cfu. At the higher dose of  $4.7 \times 10^9$  1/6 volunteers experienced diarrhoea and 2/6 had mild abdominal cramps. Bacterial shedding was seen in all volunteers at the  $5 \times 10^9$  cfu dose level from day 1 post vaccination until, 30 as per protocol, ciprofloxacin was started on day 4 after vaccination. This indicates good intestinal colonisation, which is indicative of the potential to induce a good immune response. At the two lower doses, vaccine strain was recovered from all volunteers on at least one time point following vaccination but the duration of the excretion was reduced compared to that seen at the highest dose.

At the time of filing the application, the analysis of the 40 immune responses generated by the vaccine was incomplete.

However, the IgA anti-CFA II responses in the culture supernatants of PBMNC purified from the blood of recipients of the highest dose of vaccine at day 0 (before vaccination) and days 7 and 10 post vaccination have been 5 analysed (see Figure 7). Supernatants were analysed by ELISA on assay plates coated with purified CFA II antigen. The OD values observed from the day 7 and day 10 samples were significantly higher than those from the pre-vaccination samples, demonstrating the induction of a 10 specific IgA response at these time points. As expected, the responses show a peak at day 7 and are reduced at day 10, consistent with the homing of primed IgA secreting B-cells from the blood to the mucosal effector sites of the Gut Associated Lymphoid Tissue.

15

**Conclusions:**

The attenuated live strain of ETEC ( $\Delta$ aeroC/ $\Delta$ ompC/ $\Delta$ ompF) has been shown to be well tolerated in healthy adult volunteers and to colonise the intestine in a manner 20 consistent with its utility as an oral vaccine to protect against travellers diarrhoea. It has also been demonstrated to elicit a specific mucosal immune response.

25

30

References

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CLAIMS

1. A bacterium attenuated by a non-reverting mutation  
in each of the *aroC* gene, the *ompF* gene and the  
5       *ompC* gene.
2. A bacterium according to claim 1 which infects by  
the oral route.
- 10 3. A bacterium according to claim 1 which is from the  
genera *Escherichia*, *Salmonella*, *Vibrio*,  
*Haemophilus*, *Neisseria*, *Yersinia*, *Bordetella* or  
*Brucella*.
- 15 4. A bacterium according to claim 3 which is a strain  
of *Escherichia coli*, *Salmonella typhimurium*,  
*Salmonella typhi*, *Salmonella enteritidis*,  
*Salmonella choleraesuis*, *Salmonella dublin*,  
*Haemophilus influenzae*, *Neisseria gonorrhoeae*,  
20       *Yersinia enterocolitica*, *Bordetella pertussis* or  
*Brucella abortus*.
5. A bacterium according to claim 4 which is a strain  
of enterotoxigenic *E.coli* (ETEC).
- 25 6. A bacterium according to any one of the preceding  
claims which is further attenuated by a mutation in  
a fourth gene.

7. A bacterium according to claim 6 wherein the fourth gene is *aroA*, *aroD*, *aroE*, *pur*, *htrA*, *galE*, *cya*, *crp*, *phoP* or *surA*.
- 5 8. A bacterium according to any one of the preceding claims, wherein the mutation in each gene is a defined mutation.
9. A bacterium according to any one of the preceding 10 claims, wherein the mutation in each gene is deletion of the entire coding sequence.
10. A bacterium according to any one of the preceding claims which has been genetically engineered to 15 express a heterologous antigen.
11. A bacterium according to claim 10, wherein expression of the antigen is driven by the *nirB* promoter or the *htrA* promoter.
- 20 12. A vaccine comprising a bacterium as defined in any one of the preceding claims and a pharmaceutically acceptable carrier or diluent.
- 25 13. A bacterium as defined in any one of claims 1 to 11 for use in a method of vaccinating a human or animal.
14. An enterotoxigenic *E.coli* cell attenuated by a non-reverting mutation in each of the *aroC* gene, the 30

*ompF* gene and the *ompC* gene, for use in a method of vaccinating a human or animal against diarrhoea.

15. Use of a bacterium as defined in any one of claims  
5 1 to 11 for the manufacture of a medicament for  
vaccinating a human or animal.

16. A method of raising an immune response in a  
10 mammalian host, which comprises administering to  
the host a bacterium attenuated by a non-reverting  
mutation in each of the *aroC* gene, the *ompF* gene  
and the *ompC* gene.

15

0093522-013101

Fig. 1.

- Primer A, includes restriction site 1
- ▨— Primer B, includes restriction site 2
- ▨▨— Primer C, includes restriction sites 3 and 4
- ▨▨— Primer D, includes restriction sites 3 and 4

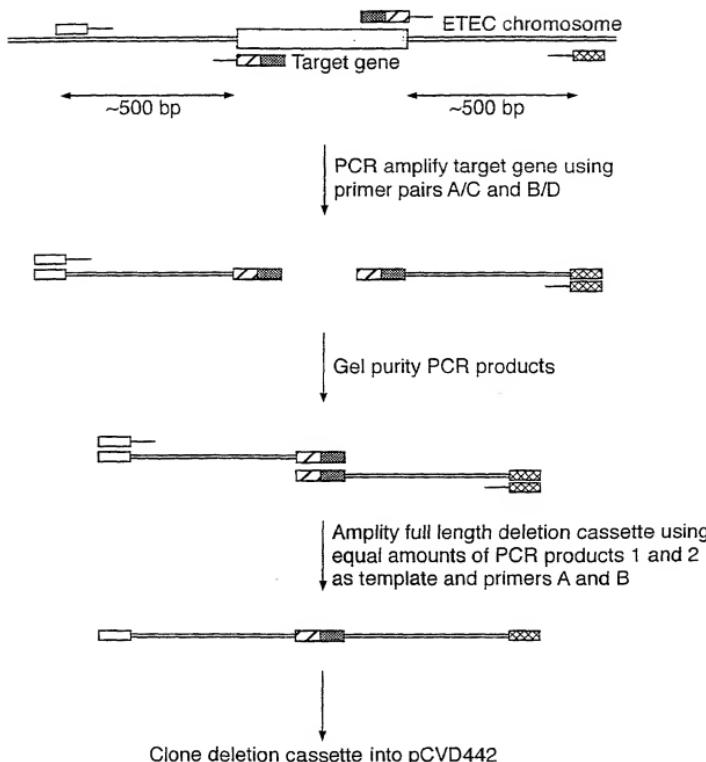


Fig. 2.

<i>aroC</i>	AAACACAACAATAACGGAGCGTGTGATG...-TAAAAATGAATAAAAACCGCGATTG CG
w.t.	<u>AAACACAACAATAACGGAGCGCCTCGAGCTGATGCTGATAAAAATGATAAAAACCGCGATTG CG</u>
deletion	
<i>htrA</i>	TGTTAACATCGAGAXTGAAAATACATGAA...-AGTAATCTCCCTCAACCCCTTCCT GAA
w.t.	<u>TGTTAACATCGAGAXTGAAAATACCTCGAGCTGAGCTAGACTCCGCTCAACCCCTTCCT GAA</u>
deletion	
<i>ompC</i>	ATATAACAGAGGGTTAAATAACATGAAA...-CAGTTCTAA TCTCGATTGATATCGAAC
w.t.	<u>ATATAACAGAGGGTTAAATAACGCTTAAGCCTCGAGTCAA TCTCGATTGATATCGAAC</u>
deletion	
<i>ompF</i>	AAACCATGAGGGTAATAAAAATAATGATAAGGC...-CCAGTTCTAA TAGCACACCTTTGTTA
w.t.	<u>AAACCATGAGGGTAATAAAAATAAGCTTAAGCCTCGAGTCAA TCTCGATTGATATCGAAC</u>
deletion	
<i>ompR</i>	CGAACCTTGGGAGTACAAACAAATGCAA...-AAGCATGAGGCCATTGCGCTTCGCCA
w.t.	<u>CGAACCTTGGGAGTACAAACAGCTAAAGCGCATGCGA GGCGATTGCGCTTCGCCA</u>
deletion	

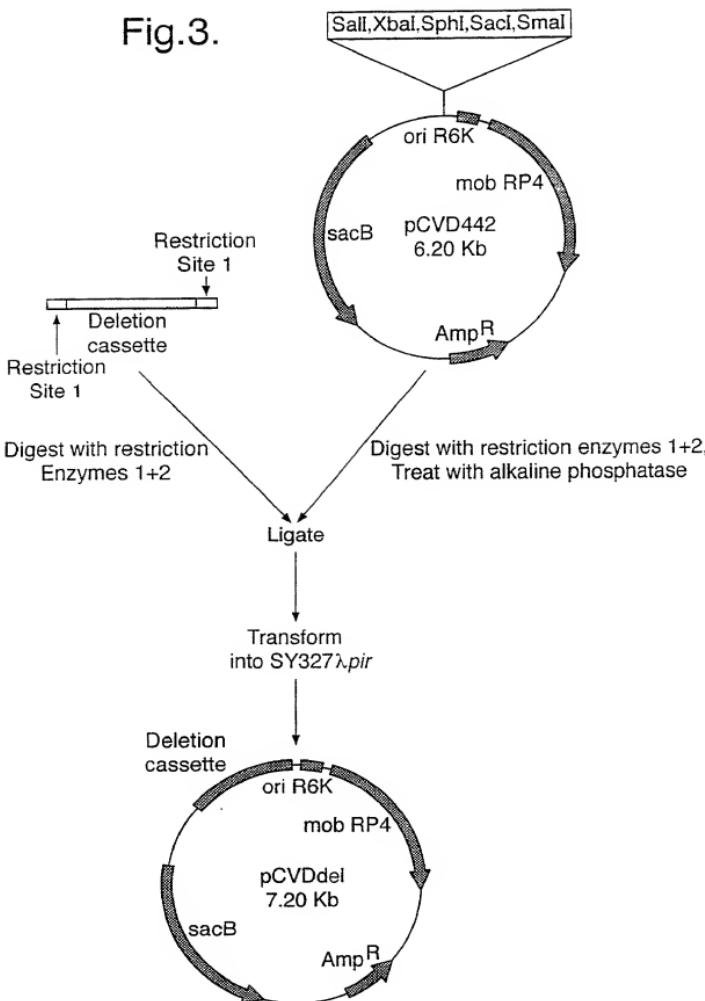
**Bold – Stop and start codons***Italics – restriction enzyme sites introduced*Underlined – primer binding sites

Lower case – extra n.t added to primers to avoid primer dimer formation

→ wild type gene

N.B. *aroC* deletion removes 16 n.t. 3' to the stop codon

Fig.3.



4/6

Fig.4.

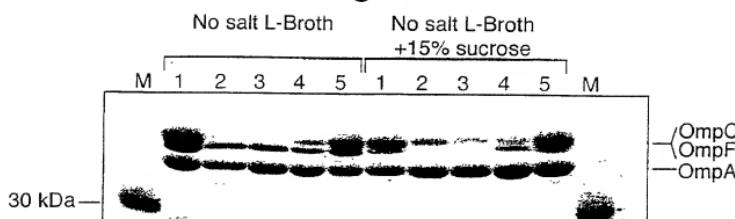
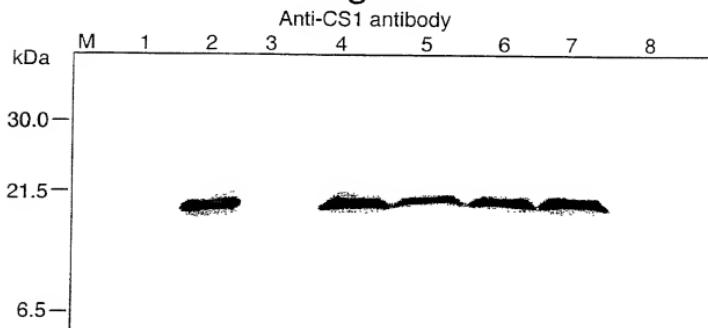


Fig.5.



Anti-CS3 antibody

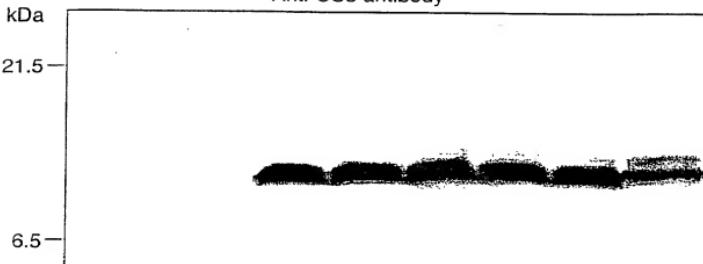


Fig. 6.

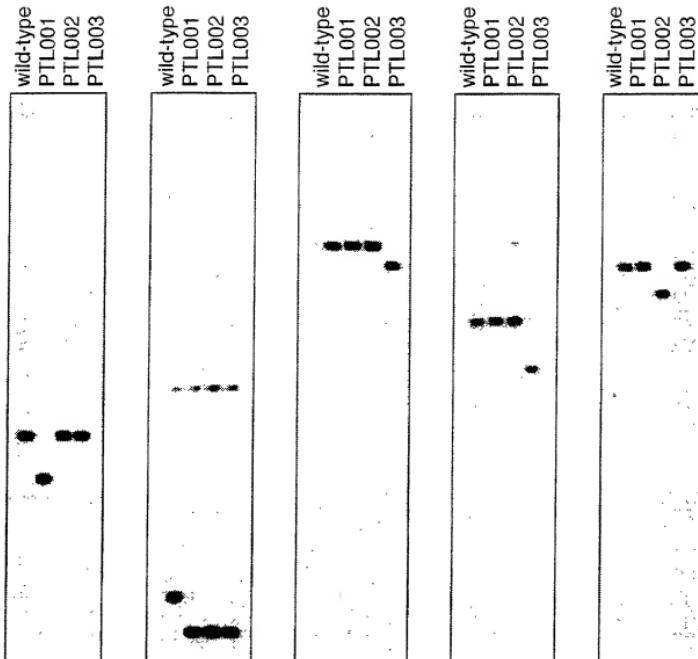
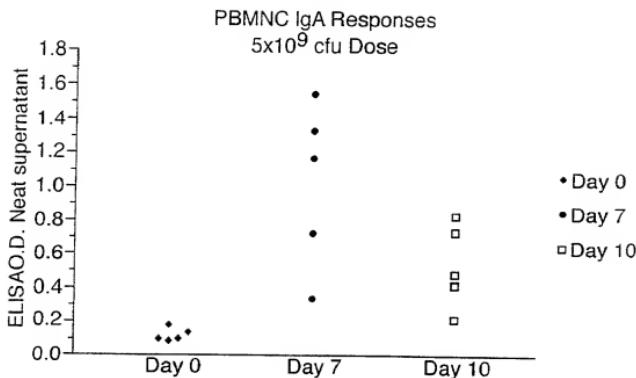


Fig.7.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: PEPTIDE THERAPEUTICS LIMITED
- (B) STREET: 100 Fulbourn Road
- (C) CITY: Cambridge
- 10 (D) STATE: not applicable
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): CB1 9PT

10

## (ii) TITLE OF INVENTION: ATTENUATED BACTERIA USEFUL IN VACCINES

15

## (iii) NUMBER OF SEQUENCES: 6

## (iv) COMPUTER READABLE FORM:

20

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 1690 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

35

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

100310-15264560

(A) ORGANISM: aroC of E.coli

(ix) FEATURE:

(A) NAME/KEY: CDS

5 (B) LOCATION:492..1562

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

	GTGAGCGCG	TGGATATCTC	TCCAGACGCG	CTGGCGGTTG	CTGAAACGAA	CATCGAAGAA	60
10	CACGGTCTGA	TCCACAACTG	CATTCCGATT	CGTTCGGATC	TGTTCCGCGA	CTTCCGAAA	120
	GTGCACTACG	ACCTGATTG	CACTAACCG	CGTGTGTCG	ATGCCAAGAT	ATGTCGACC	180
	TGCAAACAA	TACCGCGAG	AGCCGGAAC	GGGCGCTGGCA	TCTGGCACTG	ACGGCGTGA	240
	ACTGACCGT	CGCATTCG	GTAACGCGGC	AGATTACCTT	GCTGATGATG	GGGTGTTGAT	300
	TTTGAGTC	GGCAACAGCA	TGGTACATCT	TATGGAAACA	TATCCGGATG	TTCCGTTCAC	360
15	CTGGCTGGAG	TTTGATAACG	GCGGGGATGG	TGTGTTTAG	CTCACCAAAG	AGCAGCTTAT	420
	TGCGGACAGA	GAACATTTCG	CGATTTATAA	AGATTAAGTA	AAACAGCAAA	CACAACAATA	480
	ACGGAGCCGT	G ATG GCT	GGA AAC ACA ATT	GGA CAA CTC TTT	CGC GTA ACC		530
	Met Ala	Gly Asn	Thr Ile	Gly Gln	Leu Phe	Arg Val Thr	
	1	5	10				
20	ACC TTC GGC GAA	TCG CAC	GGG CTG	GCG CTC	GGC TGC	ATC GTC	578
	Thr Phe	Gly Ser	His Gly	Leu Ala	Leu Gly	Cys Ile	
	15	20	25				
	Val Pro	Pro Gly	Ile Pro	Leu Thr	Glu Ala	Asp Leu	
25	GTT CGG CCA GGC ATT	CCG CTG	ACG GAA	GCG GAC	CTG CAA	CAT GAC	626
	Val Pro	Pro Gly	Ile Pro	Leu Thr	Glu Ala	Asp Leu	
	30	35	40	45			
	GAC CGT CGT CGC CCT	GGG ACA	TCG CGC	TAT ACC	ACC CAG	CGC CGC GAG	674
30	Asp Arg	Arg Arg	Pro Gly	Thr Ser	Arg Tyr	Thr Gln	
	50	55	60				
	CCG GAT CAG GTC AAA ATT	CTC TCC	GGT GTT	TTT GAA	GGC GTT	ACT ACC	722
	Pro Asp	Gln Val	Lys Ile	Leu Ser	Gly Val	Phe Glu	
35	65	70	75				
	GGC ACC AGC ATT	GGC TTG	TTG ATC	GAA AAC	ACT GAC	CAG CGC TCT CAG	770

Gly Thr Ser Ile Gly Leu Leu Ile Glu Asn Thr Asp Gln Arg Ser Gin  
 80 85 90

GAT TAC AGT GCG ATT AAG GAC GTT TTC CGT CCA GGC CAT GCC GAT TAC 818  
 5 Asp Tyr Ser Ala Ile Lys Asp Val Phe Arg Pro Gly His Ala Asp Tyr  
 95 100 105

ACC TAC GAA CAA AAA TAC GGT CTG CGC GAT TAT CGC GGC GGT GGA CGT 866  
 Thr Tyr Glu Gin Lys Tyr Gly Leu Arg Asp Tyr Arg Gly Gly Arg  
 10 110 115 120 125

TCT TCC GCC CGC GAA ACC GCC ATG CGC GTG GCG GCA GGA GCT ATT GCC 914  
 Ser Ser Ala Arg Glu Thr Ala Met Arg Val Ala Ala Gly Ala Ile Ala  
 130 135 140

15 AAA AAA TAT CTC GCC GAG AAA TTT GGT ATT GAA ATC CGT GGC TGC CTG 962  
 Lys Lys Tyr Leu Ala Glu Lys Phe Gly Ile Glu Ile Arg Gly Cys Leu  
 145 150 155

20 ACC CAG ATG GGC GAC ATT CCG CTG GAT ATC AAA GAC TGG TCG CAG GTC 1010  
 Thr Gln Met Gly Asp Ile Pro Leu Asp Ile Lys Asp Trp Ser Gln Val  
 160 165 170

25 GAG CAA AAT CCG TTT TTT TGC CCG GAC CCC GAC AAA ATC GAC GCG TTA 1058  
 Glu Gln Asn Pro Phe Phe Cys Pro Asp Pro Asp Lys Ile Asp Ala Leu  
 175 180 185

30 GAC GAG TTG ATG CGT GCG CTG AAA AAA GAG GGC GAC TCC ATC GGC GCT 1106  
 Asp Glu Leu Met Arg Ala Leu Lys Lys Glu Gly Asp Ser Ile Gly Ala  
 190 195 200 205

35 AAA GTC ACC GTT GTT GCC AGT GGC GTT CCT GCC GGA CTT GGC GAG CGG 1154  
 Lys Val Thr Val Val Ala Ser Gly Val Pro Ala Gly Leu Gly Glu Pro  
 210 215 220

GTC TTT GAC CGC CTG GAT GCT GAC ATC GCC CAT GCG CTG ATG AGC ATC 1202  
 Val Phe Asp Arg Leu Asp Ala Asp Ile Ala His Ala Leu Met Ser Ile

	225	230	235	
	AAC GCG GTG AAA GGC GTG GAA ATT GGC GAC GGC TTT GAC GTG GTG GCG			1250
	Asn Ala Val Lys Gly Val Glu Ile Gly Asp Gly Phe Asp Val Val Ala			
5	240	245	250	
	CTG CGC GGC AGC CAG AAC CGC GAT GAA ATC ACC AAA GAC GGT TTC CAG			1298
	Leu Arg Gly Ser Gln Asn Arg Asp Glu Ile Thr Lys Asp Gly Phe Gln			
	255	260	265	
10	AGC AAC CAT GCG GGC GGC ATT CTC GGC GGT ATC AGC AGC GGG CAG CAA			1346
	Ser Asn His Ala Gly Gly Ile Leu Gly Gly Ile Ser Ser Gly Gln Gln			
	270	275	280	285
15	ATC ATT GCC CAT ATG GCG CTG AAA CCG ACC TCC AGC ATT ACC GTG CCG			1394
	Ile Ile Ala His Met Ala Leu Lys Pro Thr Ser Ser Ile Thr Val Pro			
	290	295	300	
	GGT CGT ACC ATT AAC CGC TTT GGC GAA GAA GTT GAG ATG ATC ACC AAA			1442
20	Gly Arg Thr Ile Asn Arg Phe Gly Glu Glu Val Glu Met Ile Thr Lys			
	305	310	315	
	GGC CGT CAC GAT CCC TGT GTC GGG ATC CGC GCA GTG CCG ATC GCA GAA			1490
	Gly Arg His Asp Pro Cys Val Gly Ile Arg Ala Val Pro Ile Ala Glu			
25	320	325	330	
	GCG AAT GCT GGC GAT CGT TTT ATT GGA TCA CCT GTT ACG GCA ACG GGC			1538
	Ala Asn Ala Gly Asp Arg Phe Asn Gly Ser Pro Val Thr Ala Thr Gly			
	335	340	345	
30	GCA AAA TGC CGA TGT GAA GAC TGA TATTCACGC TGTTAAAAAA TGAATAAAC			1592
	Ala Lys Cys Arg Cys Glu Asp *			
	350	355		
35	CGCGATTGCG CTGCTGGCTC TGCTTGCCAG TAGGCCAGC CTGGCAGCGA CGCCGTGGCA			1652
	AAAAATAACC CAACCTGTGC CGGGTAGCGC CAAATCGA			1690

00935/00/1301-0100

(2) INFORMATION FOR SEO ID NO: 2-

(i) SEQUENCE CHARACTERISTICS:

5                   (A) LENGTH: 356 amino acids  
                     (B) TYPE: amino acid  
                     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Gly Asn Thr Ile Gly Gln Leu Phe Arg Val Thr Thr Phe Gly

1                    5                    10                    15

15 Glu Ser His Gly Leu Ala Leu Gly Cys Ile Val Asp Gly Val Pro Pro  
20 25 30

Gly Ile Pro Leu Thr Glu Ala Asp Leu Gln His Asp Leu Asp Arg Arg

35 40 45

Arg Pro Gly Thr Ser Arg Tyr Thr Thr Gln Arg Arg Glu Pro Asp Gln

Val Lys Ile Leu Ser Gly Val Phe Glu Gly Val Thr Thr Gly Thr Ser  
25 65 70 75 80

Ile Gly Leu Leu Ile Glu Asn Thr Asp Gln Arg Ser Gln Asp Tyr Ser  
 85 90 95

30 Ala Ile Lys Asp Val Phe Arg Pro Gly His Ala Asp Tyr Thr Tyr Glu  
100 105 110

Gln Lys Tyr Gly Leu Arg Asp Tyr Arg Gly Gly Gly Arg Ser Ser Ala  
115 120 125

35 Arg Glu Thr Ala Met Arg Val Ala Ala Gly Ala Ile Ala Lys Lys Tyr  
           130               135               140

Leu Ala Glu Lys Phe Gly Ile Glu Ile Arg Gly Cys Leu Thr Gln Met  
145 150 155 160

Gly Asp Ile Pro Leu Asp Ile Lys Asp Trp Ser Gln Val Glu Gln Asn  
5 165 170 175

Pro Phe Phe Cys Pro Asp Pro Asp Lys Ile Asp Ala Leu Asp Glu Leu  
180 185 190

10 Met Arg Ala Leu Lys Lys Glu Gly Asp Ser Ile Gly Ala Lys Val Thr  
195 200 205

Val Val Ala Ser Gly Val Pro Ala Gly Leu Gly Glu Pro Val Phe Asp  
15 210 215 220

Arg Leu Asp Ala Asp Ile Ala His Ala Leu Met Ser Ile Asn Ala Val  
225 230 235 240

Lys Gly Val Glu Ile Gly Asp Gly Phe Asp Val Val Ala Leu Arg Gly  
20 245 250 255

Ser Gln Asn Arg Asp Glu Ile Thr Lys Asp Gly Phe Gln Ser Asn His  
260 265 270

25 Ala Gly Gly Ile Leu Gly Gly Ile Ser Ser Gly Gln Gln Ile Ile Ala  
275 280 285

His Met Ala Leu Lys Pro Thr Ser Ser Ile Thr Val Pro Gly Arg Thr  
290 295 300

Ile Asn Arg Phe Gly Glu Glu Val Glu Met Ile Thr Lys Gly Arg His  
305 310 315 320

35 Asp Pro Cys Val Gly Ile Arg Ala Val Pro Ile Ala Glu Ala Asn Ala  
325 330 335

Gly Asp Arg Phe Asn Gly Ser Pro Val Thr Ala Thr Gly Ala Lys Cys

340

345

350

Arg Cys Glu Asp \*

5 355

(2) INFORMATION FOR SEQ ID NO: 3:

(1) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 1713 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *ompC* of *E.coli*

20 (ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 491..1594

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTTAACAAAGC GTTATAGTTT TTCTGTGGTA GCACAGAATA ATGAAAAGTG TGTAAGAAG 60

GGTAAAAAAA ACCGAATCGG AGGCATCCGG TTGAAATAGG GGTAACAGA CATTCAAGAA 120

30 TGAATGACGG TAATAAATAA AGTTAATGAT GATAGCGGGA GTTATTCTAG TTGCGAGTGA 180

AGGTTTTGTT TTGACATTCA GTGCTGTCAA ATACTTAAGA ATAAGTTATT GATTTTAACC 240

35 TTGAATTATT ATTGCTTGAT GTTAGGTGCT TATTTCGCCA TTCCGCAATA ATCTTAAAAA 300

GTTCCCTTGC ATTTACATTT TGAAACATCT ATAGCGATAA ATGAAACATC TTAAAAGTTT 360

	TAGTATCATA TTCTGTGG ATTATTCTGC ATTTTGCCCC AGAATGGACT TGCCGACTGA	420
	TTAATGAGGG TTAATCAGTA TGCAGTGGCA TAAAAAAGCA AATAAAGGCATA AACAGAG	480
5	GGTTAATAAC ATG AAA GTT AAA GTA CTG TCC CTC CTG GTC CCA GCT CTG Met Lys Val Lys Val Leu Ser Leu Leu Val Pro Ala Leu	529
	360 365 370	
10	CTG GTA GCA GGC GCA GCA AAC GCT GCT GAA GTT TAC AAC AAA GAC GGC Leu Val Ala Gly Ala Ala Asn Ala Ala Glu Val Tyr Asn Lys Asp Gly	577
	375 380 385	
15	AAC AAA TTA GAT CTG TAC GGT AAA GTA GAC GGC CTG CAC TAT TTC TCT Asn Lys Leu Asp Leu Tyr Gly Lys Val Asp Gly Leu His Tyr Phe Ser	625
	390 395 400	
20	GAC AAC AAA GAT GTA GAT GGC GAC CAG ACC TAC ATG CGT CTT GGC TTC Asp Asn Lys Asp Val Asp Gly Asp Gln Thr Tyr Met Arg Leu Gly Phe	673
	405 410 415	
25	AAA GGT GAA ACT CAG GTT ACT GAC CAG CTG ACC GGT TAC GGC CAG TGG Lys Gly Glu Thr Gln Val Thr Asp Gln Leu Thr Gly Tyr Gln Trp	721
	420 425 430	
30	GAA TAT CAG ATC CAG GGC AAC AGC GCT GAA AAC GAA AAC AAC TCC TGG Glu Tyr Gln Ile Gln Gly Asn Ser Ala Glu Asn Glu Asn Asn Ser Trp	769
	435 440 445 450	
35	ACC CGT GTG GCA TTC GCA GGT CTG AAA TTC CAG GAT GTG GGT TCT TTC Thr Arg Val Ala Phe Ala Gly Leu Lys Phe Gln Asp Val Gly Ser Phe	817
	455 460 465	
	GAC TAC GGT CGT AAC TAC GGC GTT TAT GAC GTA ACT TCC TGG ACC	865
35	Asp Tyr Gly Arg Asn Tyr Gly Val Val Tyr Asp Val Thr Ser Trp Thr	
	470 475 480	

	GAC GTA CTG CCA GAA TTC GGT GAC ACC TAC GGT TCT GAC AAC TTC		913
	Asp Val Leu Pro Glu Phe Gly Gly Asp Thr Tyr Gly Ser Asp Asn Phe		
485	490	495	
5	ATG CAG CAG CGT GGT AAC GGC TTC GCG ACC TAC CGT AAC ACT GAC TTC		961
	Met Gln Gln Arg Gly Asn Gly Phe Ala Thr Tyr Arg Asn Thr Asp Phe		
500	505	510	
10	TTC GGT CTG GTT GAC GGC CTG AAC TTT GCT GTT CAG TAC CAG GGT AAA		1009
	Phe Gly Leu Val Asp Gly Leu Asn Phe Ala Val Gln Tyr Gln Gly Lys		
515	520	525	530
15	AAC GGC AAC CCA TCT GGT GAA GGC TTT ACT AGT GGC GTA ACT AAC AAC		1057
	Asn Gly Asn Pro Ser Gly Glu Gly Phe Thr Ser Gly Val Thr Asn Asn		
535	540	545	
20	GGT CGT GAC GCA CTG CGT CAA AAC GGC GAC GGC GTC GGC GGT TCT ATC		1105
	Gly Arg Asp Ala Leu Arg Gln Asn Gly Asp Gly Val Gly Ser Ile		
550	555	560	
25	ACT TAT GAT TAC GAA GGT TTC GGT ATC GGT GGT GCG ATC TCC AGC TCC		1153
	Thr Tyr Asp Tyr Glu Gly Phe Gly Ile Gly Ala Ile Ser Ser Ser		
565	570	575	"
30	AAA CGT ACT GAT GCT CAG AAC ACC GCT GCT TAC ATC GGT AAC GGC GAC		1201
	Lys Arg Thr Asp Ala Gln Asn Thr Ala Ala Tyr Ile Gly Asn Gly Asp		
580	585	590	
35	CGT GCT GAA ACC TAC ACT GGT GGT CTG AAA TAC GAC GCT AAC AAC ATC		1249
	Arg Ala Glu Thr Tyr Thr Gly Leu Lys Tyr Asp Ala Asn Asn Ile		
595	600	605	610
40	TAC CTG GCT CAG TAC ACC CAG ACC TAC AAC GCA ACT CGC GTA GGT		1297
	Tyr Leu Ala Ala Gln Tyr Thr Gln Thr Tyr Asn Ala Thr Arg Val Gly		
615	620	625	
45	TCC CTG GGT TGG GCG AAC AAA GCA CAG AAC TTC GAA GCT GTT GCT CAG		1345

	Ser Leu Gly Trp Ala Asn Lys Ala Gln Asn Phe Glu Ala Val Ala Gln			
	630	635	640	
	TAC CAG TTC GAC TTC GGT CTG CGT CCG TCC CTG GCT TAC CTG CAG TCT			1393
5	Tyr Gln Phe Asp Phe Gly Leu Arg Pro Ser Leu Ala Tyr Leu Gln Ser	645	650	655
	AAA GGT AAA AAC CTG GGT CGT GGC TAC GAC GAC GAA GAT ATC CTG AAA			1441
	Lys Gly Lys Asn Leu Gly Arg Gly Tyr Asp Asp Glu Asp Ile Leu Lys			
10	660	665	670	
	TAT GTT GAT GTT GGT GCT ACC TAC TAC TTC AAC AAA AAC ATG TCC ACC			1489
	Tyr Val Asp Val Gly Ala Thr Tyr Tyr Phe Asn Lys Asn Met Ser Thr			
	675	680	685	690
15	TAC GTT GAC TAC AAA ATC AAC CTG CTG GAC GAC AAC CAG TTC ACT CGT	1537		
	Tyr Val Asp Tyr Lys Ile Asn Leu Leu Asp Asp Asn Gln Phe Thr Arg			
	695	700	705	
20	GAC GCT GGC ATC AAC ACT GAT AAC ATC GTA GCT CTG GGT CTG GTT TAC	1585		
	Asp Ala Gly Ile Asn Thr Asp Asn Ile Val Ala Leu Gly Leu Val Tyr			
	710	715	720	
	CAG TTC TAA TCTCGATTGA TATCGAACAA GGGCCTGCGG GCCCTTTTT			1634
25	Gln Phe *	725		
	CATTGTTTC AGCGTACAAA CTCAGTTTT TGTTGTACTC TTGGCGACCGT TCGCATGAGG			1694
30	ATAATCACGT ACGGAAATA			1713

## (2) INFORMATION FOR SEQ ID NO: 4:

- 35           (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 367 amino acids
  - (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

5

Met Lys Val Lys Val Leu Ser Leu Leu Val Pro Ala Leu Leu Val Ala  
1 5 10 15

Gly Ala Ala Asn Ala Ala Glu Val Tyr Asn Lys Asp Gly Asn Lys Leu  
10 20 25 30

Asp Leu Tyr Gly Lys Val Asp Gly Leu His Tyr Phe Ser Asp Asn Lys  
35 40 45

15 Asp Val Asp Gly Asp Gln Thr Tyr Met Arg Leu Gly Phe Lys Gly Glu  
50 55 60

Thr Gln Val Thr Asp Gln Leu Thr Gly Tyr Gln Trp Glu Tyr Gln  
65 70 75 80

20 Ile Gln Gly Asn Ser Ala Glu Asn Glu Asn Asn Ser Trp Thr Arg Val  
- 85 90 95

Ala Phe Ala Gly Leu Lys Phe Gln Asp Val Gly Ser Phe Asp Tyr Gly  
25 100 105 110

Arg Asn Tyr Gly Val Val Tyr Asp Val Thr Ser Trp Thr Asp Val Leu  
115 120 125

30 Pro Glu Phe Gly Gly Asp Thr Tyr Gly Ser Asp Asn Phe Met Gln Gln  
130 135 140

Arg Gly Asn Gly Phe Ala Thr Tyr Arg Asn Thr Asp Phe Phe Gly Leu  
145 150 155 160

35 Val Asp Gly Leu Asn Phe Ala Val Gln Tyr Gln Gly Lys Asn Gly Asn  
165 170 175

Pro Ser Gly Glu Gly Phe Thr Ser Gly Val Thr Asn Asn Gly Arg Asp  
180 185 190

Ala Leu Arg Gln Asn Gly Asp Gly Val Gly Gly Ser Ile Thr Tyr Asp  
5 195 200 205

Tyr Glu Gly Phe Gly Ile Gly Gly Ala Ile Ser Ser Ser Lys Arg Thr  
210 215 220

10 Asp Ala Gln Asn Thr Ala Ala Tyr Ile Gly Asn Gly Asp Arg Ala Glu  
225 230 235 240

Thr Tyr Thr Gly Gly Leu Lys Tyr Asp Ala Asn Asn Ile Tyr Leu Ala  
245 250 255

15 Ala Gln Tyr Thr Gln Thr Tyr Asn Ala Thr Arg Val Gly Ser Leu Gly  
260 265 270

Trp Ala Asn Lys Ala Gln Asn Phe Glu Ala Val Ala Gln Tyr Gln Phe  
20 275 280 285

Asp Phe Gly Leu Arg Pro Ser Leu Ala Tyr Leu Gln Ser Lys Gly Lys  
290 295 300

25 Asn Leu Gly Arg Gly Tyr Asp Asp Glu Asp Ile Leu Lys Tyr Val Asp  
305 310 315 320

Val Gly Ala Thr Tyr Tyr Phe Asn Lys Asn Met Ser Thr Tyr Val Asp  
325 330 335

30 Tyr Lys Ile Asn Leu Leu Asp Asp Asn Gln Phe Thr Arg Asp Ala Gly  
340 345 350

Ile Asn Thr Asp Asn Ile Val Ala Leu Gly Leu Val Tyr Gln Phe \*

35 355 360 365

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1808 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (vi) ORIGINAL SOURCE:

(A) ORGANISM: OMRE of *E. coli*

(ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 457 1545

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 AAAACTAATC CGCATCTTAA TGGGGATTG GTTTTCTT AGCTTAATGC ACAATTCCA 60

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CACTTTACG GTAGCGAAAC GTTAGTTTGA ATGGAAAGAT GCCTGCAGAC ACATAAAGAC 360

ACCAAACCTT CATCAATAGT TCCGTAAATT TTATTCGACA GAACTTATTC ACCGGACTCC 420

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5

	CTG GCA GTG ATC GTC CCT GCT CTG TTA GTA GCA GGT ACT GCA AAC GCT			522
Leu Ala Val Ile Val Pro Ala Leu Leu Val Ala Gly Thr Ala Asn Ala				
10		15	20	
5	GCA GAA ATC TAT AAC AAA GAT GGC AAC AAA GTA GAT CTG TAC GGT AAA			570
Ala Glu Ile Tyr Asn Lys Asp Gly Asn Lys Val Asp Leu Tyr Gly Lys				
25		30	35	
10	GCT GTT GGT CTG CAT TAT TTT TCC AAG GGT AAC GGT GAA AAC AGT TAC			618
Ala Val Gly Leu His Tyr Phe Ser Lys Gly Asn Gly Glu Asn Ser Tyr				
40		45	50	
15	GGT GGC AAT GGC GAC ATG ACC TAT GCC CGT CTT GGT TTT AAA GGG GAA			666
Gly Gly Asn Gly Asp Met Thr Tyr Ala Arg Leu Gly Phe Lys Gly Glu				
55		60	65	70
20	ACT CAA ATC AAT TCC GAT CTG ACC GGT TAT GGT CAG TGG GAA TAT AAC			714
Thr Gln Ile Asn Ser Asp Leu Thr Gly Tyr Gly Gln Trp Glu Tyr Asn				
75		80	85	
25	TTC CAG GGT AAC AAC TCT GAA GGC GCT GAC GCT CAA ACT GGT AAC AAA			762
Phe Gln Gly Asn Asn Ser Glu Gly Ala Asp Ala Gln Thr Gly Asn Lys				
90		95	100	
30	ACG CGT CTG GCA TTC GCG GGT CTT AAA TAC GCT GAC GTT GGT TCT TTC			810
Thr Arg Leu Ala Phe Ala Gly Leu Lys Tyr Ala Asp Val Gly Ser Phe				
105		110	115	
35	GAT TAC GGC CGT AAC TAC GGT GTG GTT TAT GAT GCA CTG GGT TAC ACC			858
Asp Tyr Gly Arg Asn Tyr Gly Val Val Tyr Asp Ala Leu Gly Tyr Thr				
120		125	130	
35	GAT ATG CTG CCA GAA TTT GGT GGT GAT ACT GCA TAC AGC GAT GAC TTC			906
Asp Met Leu Pro Glu Phe Gly Gly Asp Thr Ala Tyr Ser Asp Asp Phe				
135		140	145	150
35	TTC GTT GGT CGT GTT GGC GGC GTT GCT ACC TAT CGT AAC TCC AAC TTC			954

Phe Val Gly Arg Val Gly Gly Val Ala Thr Tyr Arg Asn Ser Asn Phe  
 155                    160                    165

TTT GGT CTG GTT GAT GGC CTG AAC TTC GCT GTT CAG TAC CTG GGT AAA      1002  
 5 Phe Gly Leu Val Asp Gly Leu Asn Phe Ala Val Gln Tyr Leu Gly Lys  
 170                    175                    180

AAC GAG CGT GAC ACT GCA CGC CGT TCT AAC GGC GAC GGT GTT GGC GGT      1050  
 Asn Glu Arg Asp Thr Ala Arg Arg Ser Asn Gly Asp Gly Val Gly Gly  
 10                    185                    190                    195

TCT ATC AGC TAC GAA TAC GAA GGC TTT GGT ATC GTT GGT GCT TAT GGT      1098  
 Ser Ile Ser Tyr Glu Tyr Glu Gly Phe Gly Ile Val Gly Ala Tyr Gly  
 15                    200                    205                    210

GCA GCT GAC CGT ACC AAC CTG CAA GAA GCT CAA CCT CTT GGC AAC GGT      1146  
 Ala Ala Asp Arg Thr Asn Leu Gln Glu Ala Gln Pro Leu Gly Asn Gly  
 215                    220                    225                    230

AAA AAA GCT GAA CAG TGG GCT ACT GGT CTG AAG TAC GAC GCG AAC AAC      1194  
 Lys Lys Ala Glu Gln Trp Ala Thr Gly Leu Lys Tyr Asp Ala Asn Asn  
 20                    235                    240                    245

ATC TAC CTG GCA GCG AAC TAC GGT GAA ACC CGT AAC GCT ACG CCG ATC      1242  
 25 Ile Tyr Leu Ala Ala Asn Tyr Gly Gln Thr Arg Asn Ala Thr Pro Ile  
 250                    255                    260

ACT AAT AAA TTT ACA AAC ACC AGC GGC TTC GCC AAC AAA ACG CAA GAC      1290  
 Thr Asn Lys Phe Thr Asn Thr Ser Gly Phe Ala Asn Lys Thr Gln Asp  
 30                    265                    270                    275

GTT CTG TTA GTT GCG CAA TAC CAG TTC GAT TTC GGT CTG CGT CCG TCC      1338  
 Val Leu Leu Val Ala Gln Tyr Gln Phe Asp Phe Gly Leu Arg Pro Ser  
 35                    280                    285                    290

ATC GCT TAC ACC AAA TCT AAA GCG AAA GAC GTA GAA GGT ATC GGT GAT      1386  
 Ile Ala Tyr Thr Lys Ser Lys Ala Lys Asp Val Glu Gly Ile Gly Asp

295	300	305	310	
				1434
	GTT GAT CTG GTG AAC TAC TTT GAA GTG GGC GCA ACC TAC TAC TTC AAC Val Asp Leu Val Asn Tyr Phe Glu Val Gly Ala Thr Tyr Tyr Phe Asn			
5	315	320	325	
				1482
	AAA AAC ATG TCC ACC TAT GTT GAC TAC ATC ATC AAC CAG ATC GAT TCT Lys Asn Met Ser Thr Tyr Val Asp Tyr Ile Ile Asn Gln Ile Asp Ser			
	330	335	340	
10				
	GAC AAC AAA CTG GGC GTA GGT TCA GAC GAC ACC GTT GCT GTG GGT ATC Asp Asn Lys Leu Gly Val Gly Ser Asp Asp Thr Val Ala Val Gly Ile			1530
	345	350	355	
15	GTT TAC CAG TTC TAA TAGCACACCT CTTTGTTAAA TGCGAAAAA ACAGGACTTT Val Tyr Gln Phe *			1585
	360			
20	GGTCCTGTTT TTTTATACC TTCCAGAGCA ATCTCACGTC TTGCAAAAC AGCCTGCGTT			1645
	TTCATCAGTA ATAGTTGGAA TTTTGTTAAAT CTCCCGTTAC CCTGATAGCG GACTTCCCTT			1705
	CTGTAACCAT AATGGAACCT CGTCATGTTT GAGAACATTA CCGCCGCTCC TGCCGACCCG			1765
25	ATTCTGGGCC TGCCCGATCT GTTTCGTGCC GATGAACGTC CCG			1808

## (2) INFORMATION FOR SEQ ID NO: 6:

- 30           (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 362 amino acids.
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- 35           (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Met Lys Arg Asn Ile Leu Ala Val Ile Val Pro Ala Leu Leu Val  
1 5 10 15

Ala Gly Thr Ala Asn Ala Ala Glu Ile Tyr Asn Lys Asp Gly Asn Lys  
5 20 25 30

Val Asp Leu Tyr Gly Lys Ala Val Gly Leu His Tyr Phe Ser Lys Gly  
35 40 45

10 Asn Gly Glu Asn Ser Tyr Gly Gly Asn Gly Asp Met Thr Tyr Ala Arg  
50 55 60

Leu Gly Phe Lys Gly Glu Thr Gln Ile Asn Ser Asp Leu Thr Gly Tyr  
65 70 75 80

15 Gly Gln Trp Glu Tyr Asn Phe Gln Gly Asn Asn Ser Glu Gly Ala Asp  
85 90 95

Ala Gln Thr Gly Asn Lys Thr Arg Leu Ala Phe Ala Gly Leu Lys Tyr  
20 100 105 110

Ala Asp Val Gly Ser Phe Asp Tyr Gly Arg Asn Tyr Gly Val Val Tyr  
115 120 125

25 Asp Ala Leu Gly Tyr Thr Asp Met Leu Pro Glu Phe Gly Gly Asp Thr  
130 135 140

Ala Tyr Ser Asp Asp Phe Phe Val Gly Arg Val Gly Gly Val Ala Thr  
145 150 155 160

30 Tyr Arg Asn Ser Asn Phe Phe Gly Leu Val Asp Gly Leu Asn Phe Ala  
165 170 175

Val Gln Tyr Leu Gly Lys Asn Glu Arg Asp Thr Ala Arg Arg Ser Asn  
35 180 185 190

Gly Asp Gly Val Gly Gly Ser Ile Ser Tyr Glu Tyr Glu Gly Phe Gly

195 200 205

Ile Val Gly Ala Tyr Gly Ala Ala Asp Arg Thr Asn Leu Gln Glu Ala

210 215 220

5

Gln Pro Leu Gly Asn Gly Lys Lys Ala Glu Gln Trp Ala Thr Gly Leu

225 230 235 240

Lys Tyr Asp Ala Asn Asn Ile Tyr Leu Ala Ala Asn Tyr Gly Glu Thr

10 245 250 255

Arg Asn Ala Thr Pro Ile Thr Asn Lys Phe Thr Asn Thr Ser Gly Phe

260 265 270

15 Ala Asn Lys Thr Gln Asp Val Leu Leu Val Ala Gln Tyr Gln Phe Asp  
275 280 285

Phe Gly Leu Arg Pro Ser Ile Ala Tyr Thr Lys Ser Lys Ala Lys Asp

290 295 300

20 Val Glu Gly Ile Gly Asp Val Asp Leu Val Asn Tyr Phe Glu Val Gly  
305 310 315 320

Ala Thr Tyr Tyr Phe Asn Lys Asn Met Ser Thr Tyr Val Asp Tyr Ile

25 325 330 335

Ile Asn Gln Ile Asp Ser Asp Asn Lys Leu Gly Val Gly Ser Asp Asp

340 345 350

30 Thr Val Ala Val Gly Ile Val Tyr Gln Phe \*

355 360

**RULE 63 (37 C.F.R. 1.63)**  
**DECLARATION AND POWER OF ATTORNEY**  
**FOR PATENT APPLICATION**  
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled BACTERIA ATTENUATED BY A NON-REVERTING MUTATION IN EACH OF THE AROC, OMPC AND OMPC GENES, USEFUL AS VACCINES, the specification of which (check applicable box(es)):

[ ] is attached hereto

[ ] was filed on

[x] was filed as PCT international application No. PCT/GB 99/00935 on 25<sup>th</sup> March 1999  
and (if applicable to U.S. or PCT application) was amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(a). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) or patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application.

**Prior Foreign Application(s):**

Application Number	Country	Day/Month/Year Filed
9806449.6	United Kingdom	25 <sup>th</sup> March 1998

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior applications and the national or PCT international filing date of this application.

**Prior U.S./PCT Application(s):**

Application Serial No.	Day/Month/Year Filed	Status patented, pending, abandoned
PCT/GB 99/00935	25 <sup>th</sup> March 1999	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Nixon & Vanderhye P.C., 1100 North Glebe Road, 8th Floor, Arlington, Virginia 22201-4714, telephone number (703) 816-400 (to whom all communications are to be directed), and the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Paris, 31352; Richard G. Basha, 22770; Mark E. Nushamn, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lastovo, 33149; H. Warren Burnam, Jr., 29266; Thomas E. Byrne, 32205.

1-15 ✓ 1) Inventor's Signature N.V. Date 15-01-01

Inventor's Name (typed)	Steven	Neville	CHATFIELD
	Father	Middle Initial	Family Name
			Citizenship

Residence (City) Wokingham (State/Foreign Country) Berkshire, United Kingdom G/BX

Post Office Address 545 Eskdale Road, Winnersh Triangle, Wokingham, Berkshire United Kingdom Zip Code RG41 5TU

2) Inventor's Signature \_\_\_\_\_ Date \_\_\_\_\_

Inventor's Name (typed)	_____	Middle Initial	Family Name	Citizenship
	First			

Residence (City) \_\_\_\_\_ (State/Foreign Country) \_\_\_\_\_

Post Office Address \_\_\_\_\_ Zip Code \_\_\_\_\_